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ORIGINAL ARTICLE

Contrasting spatial genetic structure in *Annona crassiflora* populations from fragmented and pristine savannas

Rosane G. Collevatti · Mariana P. C. Telles · Jacqueline S. Lima · Felipe O. Gouveia · Thannya N. Soares

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Abstract In continuous populations, fine-scale genetic structure tends to be stronger in species with restricted pollen and seed dispersal. However, habitat fragmentation and disturbances can affect genetic diversity and spatial genetic structure due to disruption in ecological processes, such as plant reproduction and seed dispersal. In this study, we compared the genetic diversity and fine-scale spatial genetic structure (SGS) in two populations of *Annona crassiflora* (Annonaceae) in a pristine savanna Reserve (ESECAE) and in a fragmented disturbed savanna area (PABE), both in Cerrado biome in Central Brazil. The analyses were based on the polymorphism at 10 microsatellite loci. Our working hypothesis was that SGS is stronger and genetic diversity is lower in population at fragmented area (PABE) than at pristine area (ESECAE). Both populations presented high levels of polymorphism and genetic diversity and showed no sign of bottleneck for both Wilcoxon sign-rank test for heterozygosity excess ($p > 0.05$) and coalescent analyses (growth parameter g not different from zero), but population at fragmented area showed higher fixation index and stronger SGS. Besides, populations are significantly differentiated ($F_{ST} = 0.239$, $R_{ST} = 0.483$, $p < 0.001$ for both). Coalescent analyses showed high historical effective population sizes for both populations, high gene flow between ESECAE and PABE and recent time to most recent common ancestor ($\sim$37 k year BP). Our results suggest that despite the high genetic diversity, fragmentation and disturbance may have been affecting populations of this species increasing mating between closely related individuals leading to high fixation index and strong SGS.

Keywords Annonaceae · Cerrado biome · Coalescence · Gene flow · Genetic diversity · Neotropical tree

Introduction

Restriction in dispersal may lead to population subdivision because "interbreeding is restricted to small distances" (Wright 1943) forming neighborhoods (Wright 1946). For continuous populations, the effective neighborhood size is equivalent to the number of reproducing individuals in a circle of radius 2σ that may include 86.5 % of the parents of individuals at the center (Wright 1946). Estimation of neighborhood size has become common in pollination biology and pollen dispersal studies, to determine the scale and effectiveness of dispersal and the balance between local genetic drift and gene dispersal (e.g. Levin and Kerster 1971; Crawford 1984). Neighborhood size may also predict population spatial genetic structure, under a restricted spatial scale (Vekemans and Hardy 2004).

Together with dispersal, available patches for establishment, competition and population dynamics determine the spatial pattern in genetic structure (Vekemans and Hardy 2004). In continuous populations, fine-scale genetic structure tends to be stronger in species with restricted pollen and seed dispersal (Born et al. 2008). However, habitat fragmentation and disturbances may affect genetic diversity (e.g. Lowe et al. 2005; Soares et al. 2008; Moreira

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R. G. Collevatti (✉) · M. P. C. Telles · J. S. Lima · F. O. Gouveia · T. N. Soares
Laboratório de Genética & Biodiversidade, Departamento de Biologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Goiás, CP 131, Goiânia, GO 74001-970, Brazil
e-mail: rosanegc68@hotmail.com

et al. 2009) and spatial genetic structure (e.g. Nason et al. 1997; Nason and Hamrick 1997; Moreira et al. 2009) due to disruption in ecological processes (Ewers and Didham 2006) such as plant reproduction and seed dispersal (see Lowe et al. 2005; Aguilar et al. 2006 for reviews).

The Cerrado biome covers nearly 22 % of the Brazilian territory (2.5 million km^2) and comprises very heterogeneous vegetation (see Silva et al. 2006 for details). Savannas correspond to ~46 % of the Cerrado biome (~1.0 million km^2) and are restricted to dystrophic soils, with low values of pH and high levels of aluminum (Furley and Ratter 1988). However, less than 50 % of Cerrado original area still remains because of agricultural expansion in the last 50 years (Klink and Machado 2005). *Annona crassiflora* Mart. (Annonaceae) is a widely distributed tree species in the savannas of the Cerrado biome in Central Brazil. The species is hermaphroditic with protogynous flowers pollinated by beetles. The giant fruits can reach up to 2 kg, and have many woody seeds that are dispersed mainly by the tapir (*Tapirus terrestris*). The edible mesocarp is also consumed by the local population of Central Brazil, playing an important role in the economy of local populations.

Herein, we compared fine-scale spatial genetic structure and genetic diversity in two populations of *A. crassiflora* in Central Brazil: one population in a pristine area and the other in a disturbed fragmented area. The analyses were based on 10 microsatellite loci and our working hypothesis was that SGS is stronger and genetic diversity is lower in the population at the fragmented area than at the pristine area due to disruption in ecological processes that affect spatial genetic structure.

Materials and methods

Study site and sampling

To analyze and compare genetic diversity and fine-scale spatial genetic structure we sampled two populations of *A. crassiflora*, in two different areas. The pristine area was located in Aguas Emendadas Ecological Reserve—ESECAE hereafter (15°35′04.7″S 47°39′59.7″W), Distrito Federal (Fig. 1), Central Brazil, a 10,547 ha of preserved savanna. The disturbed area was a savanna fragment in Padre Bernardo municipality (PABE, hereafter), dominated by adult individuals of *A. crassiflora*, 100 km far from ESECAE (Fig. 1). The region is inside the Ecological Unit 1A sensu Silva and co-workers (Silva et al. 2006), characterized by high plain plateau land with some rolling terrain dominated by dense savanna. PABE region has a very recent history of anthropogenic disturbance (since ~1930), and ~38 % of the original vegetation has been converted to pasture and crops.

For sampling, all adult individuals (104) in a 2.2 ha patch in ESECAE were mapped and expanded leaves were collected. In PABE, we found only adult individuals, thus all individuals (100) in the fragment (4.2 ha) were mapped and sampled. Because we aimed at population comparisons, we sampled only adults in both populations since PABE population had no juveniles, seedlings or saplings.

Genetic analysis

Genomic DNA extraction followed the standard CTAB 2 % procedure (Doyle and Doyle 1987). Ten microsatellite loci previously developed for *A. crassiflora* (Pereira et al. 2008) were used to genotype all sampled individuals (Supporting Information Table S1). Primers were marked with fluorescent dyes (6-FAM, HEX and NED, Applied Biosystems, CA) and microsatellite loci amplifications were performed in a 13 μL volume containing 10.0 μM of each primer, 1U Taq DNA polymerase (Phoneutria, BR), 250 μM of each dNTP, 1× reaction buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 0.25 mg of BSA and 4.5 ng of template DNA. Amplifications were performed using PE9700 thermal controller (Applied Biosystems, CA) under the following conditions: 94 °C for 5 min (one cycle); 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min (35 cycles); and 72 °C for 30 min (one cycle). The PCR products were electrophoresed on an ABI Prism 3100 automated DNA sequencer (Applied Biosystems, CA) and were sized by comparison to a 500 internal lane standard ROX (Applied Biosystems, CA), using the GeneMaper® v4.1 software (Applied Biosystems, CA).

Genetic diversity

Genetic diversity for both populations was estimated based on the expected heterozygosity (H_e) under Hardy–Weinberg equilibrium (Nei 1978). Observed heterozygosity (H_o) and fixation index (f) were also estimated to test for deviation from Hardy–Weinberg equilibrium. Departure from linkage equilibrium was verified for all pairs of loci. Analyses and randomization based tests were performed with the software FSTAT 2.9.3.2 (Goudet et al. 1996; Goudet 2002). Population polymorphism was characterized as the number of alleles per locus and allelic richness based on rarefaction method (Mousadik and Petit 1996).

We also tested whether populations are genetically differentiated using Wright's F-statistics, F_{IT}, F_{ST}, and F_{IS} (Wright 1951), obtained from an analysis of variance of allele frequencies (Weir and Cockerham 1984), implemented in the software FSTAT 2.9.3.2 (Goudet 2002). To verify the contribution of stepwise-like mutations to the genetic differentiation we estimated Slatkin's R_{ST} (Slatkin 1995) obtained from an analysis of variance of allele size.

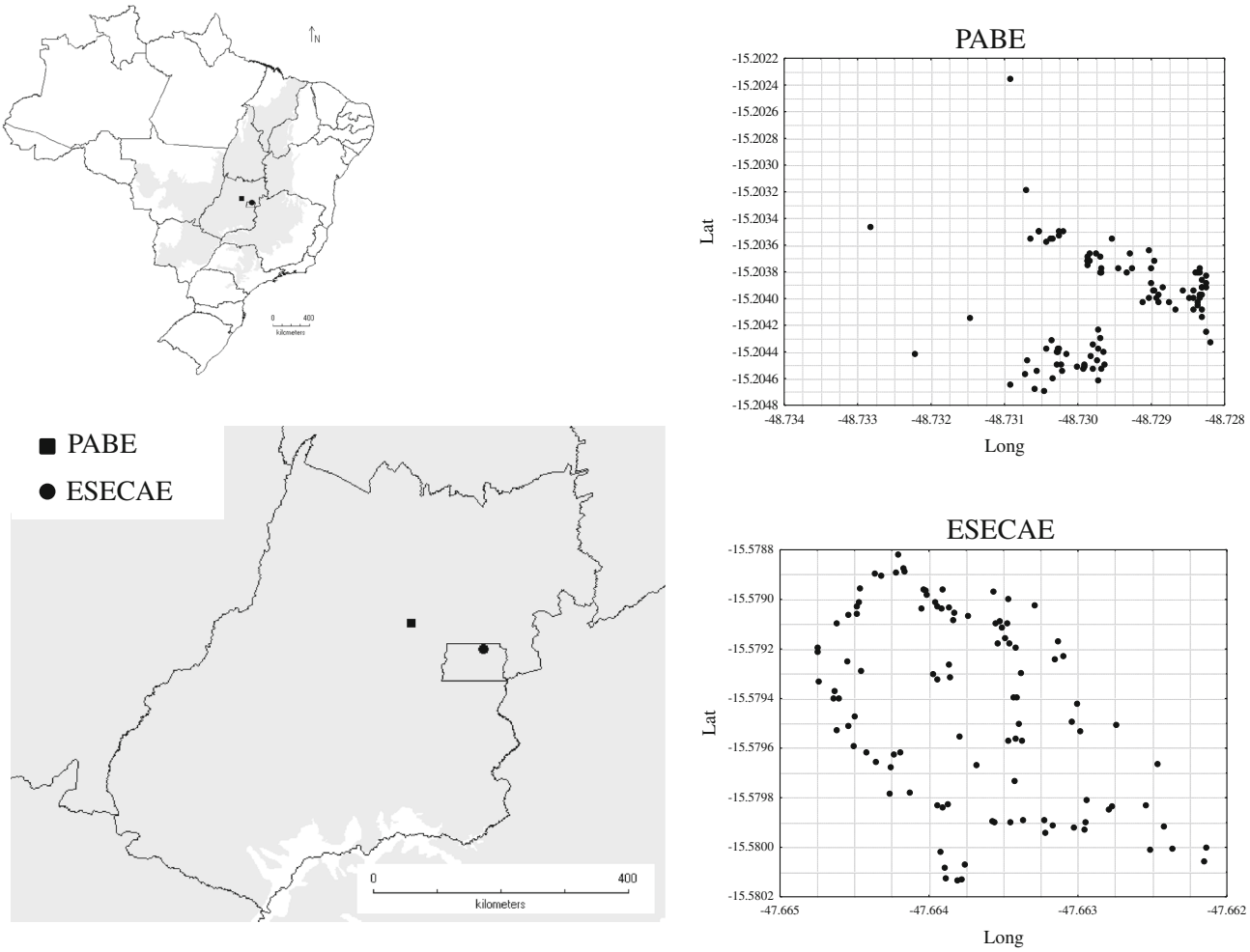

Fig. 1 Sampling localities of *Annona crassiflora* populations, and the spatial distribution of individuals in pristine savanna area (ESECAE) and in savanna fragment (PABE) in Central Brazil

Then, we tested the hypothesis that $F_{ST} = R_{ST}$ based on Hardy et al. (2006), using the software SPAGeDI (Hardy and Vekemans 2002).

Population demography

To better understand the different roles of historical demographic processes and current anthropogenic disturbances on patterns of genetic diversity, we explored changes in effective population size by estimating, for each population and the global population, the demographical parameter g (exponential growth rate, $\theta_t = \theta_{now} \exp(-gt)$, where t, the time to coalescence in mutational unit), obtained from coalescent analysis (Kingman 1982) implemented in Lamarc 2.1.8 (Kuhner 2006). We used a Bayesian approach with Markov Chain Monte Carlo (MCMC) method (Beerli and Felsenstein 2001). We also estimated the demographical parameters $\theta = 4\ \mu N_e$ (coalescent or mutation parameter for a diploid genome), and $M = 4\ N_e m/\theta$ (scaled migration rate). The analyses were run with 10 initial chains of 10,000 steps and two final chains of 100,000 steps. The chains were sampled every 100 steps following a burn-in of 10,000 steps. We used the default settings for the initial estimate of theta. The program was run three times to assess convergence and achieve ESS $\geq$ 200 (effective sample size). Combined results were obtained using Tracer v1.4.1 (Rambaut and Drummond 2007). Most probable estimates (MPE) were obtained, i.e. the highest point on the posterior probability curve for a given parameter, which is the best solution found by a Bayesian run, and also the credibility interval around the estimate of each parameter (Kuhner and Smith 2007). The effective population size was estimated from the mutation parameter θ. For this, we used the mutation rate reported for microsatellite marker in plants, 3.9×10^{-3} [95 % CI (2.9×10^{-3}–5.0×10^{-3})] mutation

per allele per generation, an intermediate value described for plants by (Udupa and Baum 2001), often quoted in the range of 10^{-2} to 10^{-4} per locus per generation (see Thuillet et al. 2002; Marriage et al. 2009). We chose this value based on the high genetic diversity in populations of *A. crassiflora* and also on the comparison of F_{ST} and R_{ST} (see results below). Time to most recent common ancestor (TMRCA) was estimated from overall θ using the above mutation rate and a generation time of 15 years (TN Soares, unpublished data).

We also analyzed the effect of reduction in population size on population genetic diversity due to bottlenecks using the Wilcoxon sign-rank test implemented in Bottleneck version 1.2.02 (Cornuet and Luikart 1997). The analyses were performed under infinite allele mutation model (IAM), stepwise mutation model (SMM) and two-phase mutation model (TPM) to verify the sensitivity of the analysis to the mutation model.

Spatial genetic structure

To understand the local spatial pattern of the individuals we performed a Multi-Distance Spatial Cluster Analysis, based on Ripley's K-function (Ripley 1981) that summarizes spatial dependence (clustering or dispersion). Observed K values larger than the expected indicate that the points are more clustered than the expected under the hypothesis of random distribution; smaller-than-expected K values suggest that the points are over-dispersed. The analyses were carried out using the software SAM v4.0 (Rangel et al. 2010).

To verify whether related genotypes are spatially structured we performed autocorrelation analyses of kinship. Pairwise kinship coefficients (F_{ij}) among individuals from a set of distance classes (see Supplemental Information Table S2) were estimated based on the Nason equation (see Loiselle et al. 1995), using the software SPAGeDi (Hardy and Vekemans 2002). To test for kinship structure, F_{ij} values were regressed on the natural logarithm of the spatial distance between individuals. Permutation tests (10.000 permutations) were performed to verify significance of kinship for each distance class and for the regression. The distance classes were defined to keep the number of pairwise comparisons within each distance interval approximately constant. Kinship values were plotted against the distance classes to visualize SGS. Standard errors (SE) were estimated by jackknife over loci. The intensity of the SGS was quantified using the parameter $S_p = -b/(1 - F_1)$, where F_1 is the average pairwise kinship coefficient between individuals of the first distance class and b is the slope of the regression on natural logarithm of the spatial distance, following Vekemans and Hardy (2004).

Genetic neighborhood and gene dispersal were estimated based on the negative relationship between spatial distance and kinship due to isolation-by-distance (Wright 1943; Rousset 1997). An iterative approach was used following Vekemans and Hardy (2004). First, genetic neighborhood (N_b) was estimated using the global regression slope b of F_{ij} on the logarithmic of the spatial distance $\ln(d_{ij})$ and the intra group F_{ij} [$N_b = (F_1 - 1)/b$]. Gene dispersal (σ_g) was estimated from this N_b ($N_b = 4\pi D_e \times \sigma_g^2$). In natural plant populations, Vekemans and Hardy (2004) showed that D_e is equivalent to 0.5–0.1 times the density of adults. Thus, in the absence of data on the variance of adult reproductive success to independently estimate effective population size, one might use this relationship. Then, N_b was iteratively estimated using the slope of a restricted regression analysis in SPAGeDI in the range of $\sigma_g < d_{ij} < 20\sigma_g$ (see Rousset 1997; Vekemans and Hardy 2004). This step was repeated until N_b estimates converged.

Results

All pairs of microsatellite loci were in linkage equilibrium (all $p > 0.05$). All loci presented high genetic diversity (Supplemental Information Table S1) and the high combined paternity exclusion (0.99999) and low probability of identity (2.03×10^{-15}) showed that the ten loci are suitable for kinship analysis. Genetic diversity and polymorphism were similar between the pristine and fragmented areas (Table 1). However, observed heterozygosity was lower than expected under Hardy–Weinberg equilibrium for the fragmented area (PABE), with fixation index significantly different from zero (Table 1, see also Supporting Information Table S1).

The two populations are significantly genetic differentiated ($F_{ST} = 0.239$, $p < 0.001$; $R_{ST} = 0.504$, $p < 0.001$) and the statistics based on allele frequency (F_{ST}) and allele size (R_{ST}) were not statistically different ($p = 0.0629$). The fixation index was also significant ($F_{IS} = 0.092$, $p < 0.001$).

Coalescent analysis showed that both populations of *A. crassiflora* have high diversity or mutation parameter (θ) leading to a large effective population size (Table 1). Using the global population coalescent parameter [$\theta = 9.821$, 95 %CI = (9.379–9.938)] we estimated TMRCA, which dated to 37,773 year BP [95 % CI = (36,100–38,222 year BP)] and effective population size, $N_e = 629.55$ [95 % CI = (601–637)]. Number of migrants per generation ($N_e m$) was higher from PABE into ESECAE [11.13 migrants per generation, 95 % CI = (0.12–13.1)] than from ESECAE into PABE [1.91 migrants per generation, 95 % CI = (0.023–1.02)].

Table 1 Comparison of genetic diversity and polymorphism between two populations of *Annona crassiflora* from Central Brazil in pristine savanna area (ESECAE) and in savanna fragment (PABE), based on 10 microsatellite loci

Population	N	A	AR ± SD	H_e ± SD	H_o ± SD	f ± SD	θ (95 % interval)	N_e (95 % interval)	g (95 % interval)
ESECAE	104	16.0	15.2 ± 7.24	0.777 ± 0.136	0.726 ± 0.152	0.057^{ns} ± 0.059	2.688 (0.064–6.436)	172.3 (65.7–968.0)	−3.179 (−39.4 to 24.1)
PAB	100	14.2	13.5 ± 7.18	0.733 ± 0.149	0.606 ± 0.189	0.173* ± 0.047	2.488 (0.040–6.226)	159.5 (41.1–639.8)	−23.3 (−85.5 to 4.9)

N, number of individuals sampled; *A*, number of alleles; *AR* allelic richness; H_e, expected heterozygosity under Hardy–Weinberg equilibrium; H_o, observed heterozygosity; *f*, inbreeding coefficient (ns, did not statistically differ from zero, $p > 0.05$; *$p < 0.001$). SD, standard deviation; θ—coalescent parameter; 95 % credibility interval around the estimate; N_e—effective population size; *g*—exponential growth parameter (all values did not differ from zero based on the credibility interval around the estimate)

Coalescent analyses also suggested that population sizes are historically constant, because the growth parameter (g) did not differ from zero for both populations (Table 1), and overall populations [$g = 0.703$, 95 % CI = (−0.032–0.866)]. In addition, both populations showed no sign of recent reduction in size followed by expansion (Wilcoxon sign-rank test $p > 0.05$ for all comparisons).

Although *A. crassiflora* was more evenly distributed in the pristine area (ESECAE, Fig. 2a), both populations presented a clumped distribution (Fig. 2b, c). The spatial genetic structure was not significant for the population in the pristine area, ESECAE (Table 2; Fig. 3) and kinship was significant only for the first class of distance (up to 25 m). The spatial structure was not significant even when a restricted regression was performed, in the range of $\sigma_g < d_{ij} < 20\sigma_g$ (Table 2). For the fragmented area, PABE, the spatial genetic structure was significant (Table 2), and kinship was significant until the third class (up to 57 m).

Neighborhood size and gene dispersal could not be estimated for the pristine area due to the lack of significant spatial genetic structure. However, in the fragmented area, PABE, *A. crassiflora* neighborhood size was ∼47 individuals, almost half of the number of individuals sampled (Table 2).

Discussion

Our results showed that, despite the high genetic diversity in both populations, the population at the fragmented and disturbed area, PABE, already shows high fixation index, which may be the outcome of selfing or biparental inbreeding most likely due to recent population size reduction. The lack of juvenile individuals may indicate low recruitment most likely due to disruption in ecological processes, such as pollination and seed dispersal. This result may also be the outcome of mating between closely related individuals that may lead to inbreeding depression and low germination or seedling survival.

Unexpectedly, despite the high anthropogenic disturbance and fragmentation PABE population presented high genetic diversity and polymorphism and no sign of recent bottleneck, most likely due to the analysis of adult individuals. The polymorphism and genetic diversity found in the presented study are similar to those found for other Neotropical tree species (e.g., Collevatti et al. 2001; Lemes et al. 2003; Jones and Hubbell 2006; Braga et al. 2007). Because anthropogenic disturbance and fragmentation is generally a recent event in evolutionary time and when considering the life cycle of the tree species, genetic effects of habitat fragmentation may be still undetectable (Collevatti et al. 2001). Despite the relatively short time since anthropogenic fragmentation in the studied area

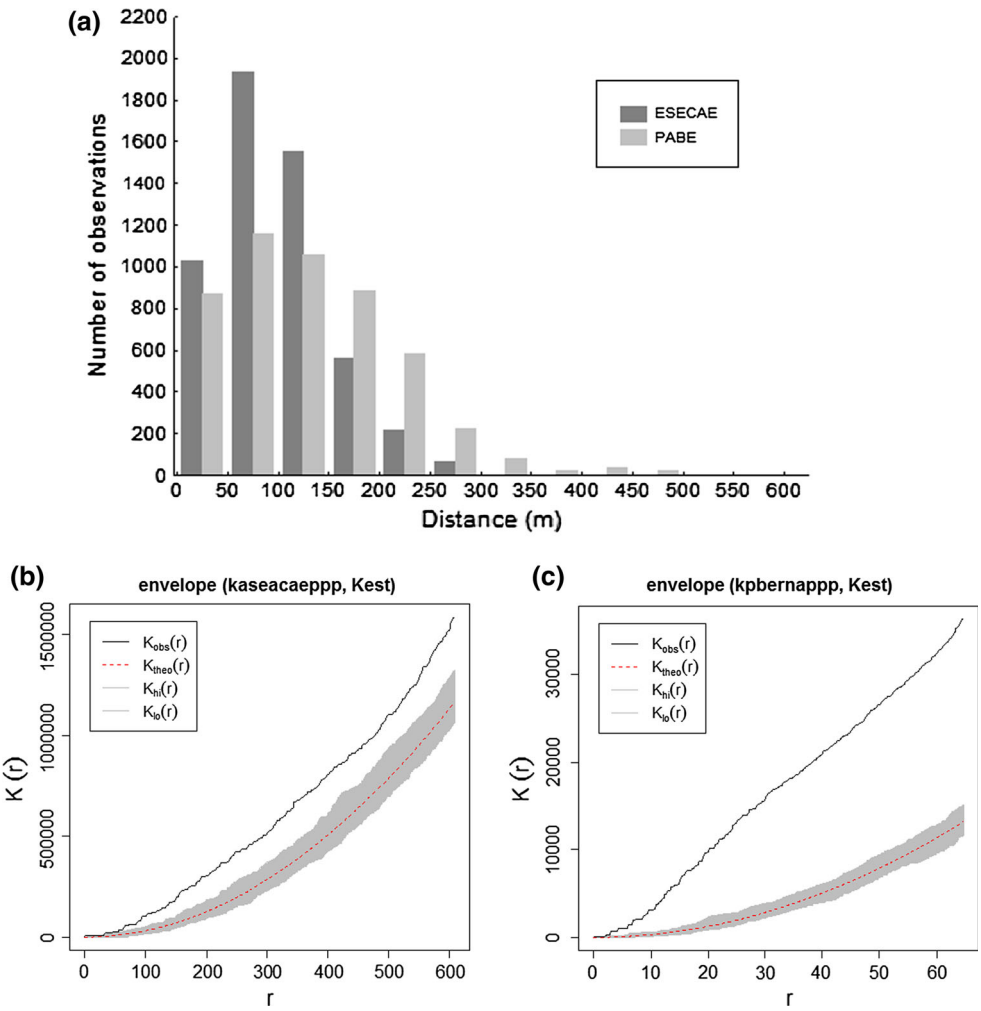

Fig. 2 Pairwise distance distribution (**a**) and spatial cluster analysis based on Ripley's K-function for *Annona crassiflora* populations in pristine savanna area (ESECAE) (**b**) and in savanna fragment (PABE) (**c**) in Central Brazil. Distance in meters. *Black thick line* observed values; *thin line* expected values for random distribution; *light gray area* confidence interval (95 %)

(~80 years), compared to the generation time of the species (15 years, 6 generation since the beginning of human disturbance), the rate of fragmentation is one of the highest reported so far for Brazilian ecosystems (Klink and Machado 2005). In addition, inferring the effects of habitat fragmentation can be especially difficult for species with high distance gene flow and long life cycle. However, high fixation index due to fragmentation and disturbance has already been reported for other Cerrado tree species in Brazil (e.g. Collevatti et al. 2001; Moreira et al. 2009; Braga and Collevatti 2011). Notwithstanding, the strength of the SGS observed in the pristine area (ESECAE) was lower than for other animal dispersed Cerrado tree species, such as *Caryocar brasiliense* (Collevatti et al. 2010; Collevatti and Hay 2011) and *Dipteryx alata* (Collevatti et al. 2010). However, the fragmented area, PABE, presented stronger SGS than *C. brasiliense* and *D. alata* and other Cerrado wind-dispersed species, such as *Tabebuia aurea* (Braga and Collevatti 2011), *T. ochracea* (Moreira et al. 2009), *Tibouchina papyrus* (Collevatti et al. 2010), *Myracrodruon urundeuva* (Gaino et al. 2010) and *Copaifera langsdorffii* (Sebbenn et al. 2011), suggesting an important effect of anthropogenic disturbance in dispersal for *A. crassiflora*.

The spatial clustering in adults suggests that the spatial distribution patterns in the studied populations are more affected by limitations to seed dispersal or by the patchy distribution of suitable microhabitat for recruitment, than by density-dependent mortality (Getzin et al. 2008). The result also suggests that the clustered patterns resulting from patchy recruitment may persist over time and that self-thinning effect due to density-dependent processes may not be important for these populations (Luo et al. 2012). Besides the lack of juveniles in fragmented population, the two populations presented differences in the SGS strength and estimates of dispersal most likely due to differences in pollen and seed dispersal leading to differences in seed shadows.

In conclusion, our results showed that *A. crassiflora* still presents high genetic diversity, despite the high fragmentation and disturbance of the savannas in Central Brazil. However, the high fixation index and lack of juvenile

Table 2 Comparison of spatial genetic structure (SGS) and gene dispersal between two populations of *Annona crassiflora* from Central Brazil in pristine savanna area (ESECAE) and in savanna fragment (PABE)

Population	N	D (ind/ha)	$F_1 \pm$ SE	$b \pm$ SE	r^2	S_p	N_b	σ^2		
								D	$0.5D$	$0.1D$
ESECAE	104	47.7	$0.0076^{*} \pm 0.0037$	$-0.0022^{ns} \pm 0.0016$	0.0003	–	–	–	–	–
PABE	100	23.8	$0.0430^{**} \pm 0.0082$	$-0.0203^{**} \pm 0.0047$	0.0306	0.0212	46.97 (38.28–61.35)	9.0 (7.0–12.0)	4.0 (3.0–6.0)	1.0 (0.7–1.2)

N, number of individuals sampled; F_1, intra group F_{ij}; b, regression slope for the logarithmic relationship; r^2, determination coefficient; S_p, strength of spatial genetic structure parameter; N_b, genetic neighborhood; σ^2, gene dispersal distance (meters) for different values of density (D); *SE* standard error. Values followed by ns did not statistically differ from zero ($p > 0.05$)

95 % CI, confidence interval at 95 % of probability

* Significant for $p < 0.05$

** Significant for $p < 0.01$

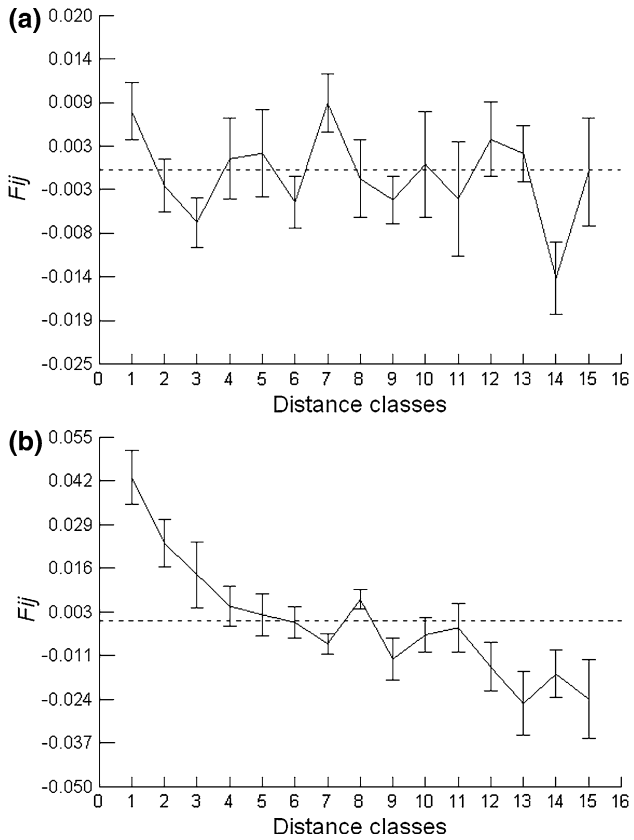

Fig. 3 Relationship between kinship ($F_{ij} \pm$ SE) and distance for *Annona crassiflora* populations in pristine savanna area (ESECAE) (**a**) and in savanna fragment (PABE) (**b**) in Central Brazil. *Black thick line* observed values; *thin lines* 95 % confidence interval. See Supplemental Information Table S2 for the spatial distance of each distance class used in spatial genetic structure analyses

individuals may be the outcomes of disturbance and fragmentation. The contrasting patterns of fine-scale genetic structure in the pristine and fragmented areas are most likely due to differences in population structure and fecundity. In addition, inbreeding may also decrease fecundity, leading to a higher kinship among progeny and, thus a higher extension of SGS in the fragmented area.

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